

Enhanced Induction of Telomerase-specific CD4⁺ T Cells Using Dendritic Cells Transfected with RNA Encoding a Chimeric Gene Product¹

Zhen Su,² Johannes Vieweg,^{2,3} Alon Z. Weizer, Philipp Dahm, Donna Yancey, Vandana Turaga, John Higgins, David Boczkowski, Eli Gilboa, and Jens Dannull

Cancer Immunotherapy Program, Division of Urology, Department of Surgery [Z. S., J. V., A. Z. W., P. D., D. Y., V. T., J. H., J. D.J., and Center for Cellular and Genetic Therapies, Department of Surgery [D. B., E. G.J., Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT

Dendritic cells (DCs) transfected with mRNA encoding human telomerase reverse transcriptase (hTERT) have been shown to represent potent inducers of CTLs and antitumor immunity. However, it has become widely accepted that not only CTLs but also CD4⁺ T helper cells are critical to the generation, as well as to the maintenance, of potent antitumor responses *in vivo*. In this study, we sought to determine whether human DCs transfected with mRNA encoding a chimeric hTERT/lysosome-associated membrane protein (LAMP-1) protein, carrying the endosomal/lysosomal sorting signal of the LAMP-1, are capable of stimulating concomitant hTERT-specific CD8⁺ and CD4⁺ T-cell responses *in vitro*. We show that processing of hTERT/LAMP-1 transcripts leads to enhanced stimulation of hTERT-specific CD4⁺ T cells and does not negatively affect intracellular generation and subsequent presentation of MHC class I epitopes, hence, generating a CTL response. These findings provide a preclinical rationale of using DCs transfected with the chimeric hTERT/LAMP-1 RNA in vaccine trials to facilitate generation of antigen-specific CD4⁺ T-cell responses that may be required to stimulate and maintain an optimal CD8⁺ CTL response *in vivo*.

INTRODUCTION

We have shown recently that autologous DCs⁴ transfected with mRNA encoding hTERT are potent inducers of CTLs and antitumor immunity (1). Immunization with hTERT mRNA-transfected DCs favors the induction of class I-restricted T-cell responses because the transfected mRNA is translated into protein in the cytoplasm. It is well known that endogenously synthesized antigens that do not gain access to the endocytic pathway are presented on MHC class I molecules. Although class I-restricted CTLs represent a major effector arm of the immune system, it has become evident that both tumor-specific CTLs and CD4⁺ T helper cell epitopes are required to elicit potent antitumor immunity (2). In this context, it has been suggested that CD4⁺ T-cell help plays an important role in the effector phase at the tumor site by enhancing the local expansion of the activated/effectector tumor-specific CTLs (3). In addition, it is well established that the efficient induction and persistence of MHC class I-restricted CTLs *in vivo* requires the participation of antigen-specific, MHC class II-restricted CD4⁺ T cells (2, 4). Finally, it was shown that CD4⁺ T cells can influence the antitumor CTL response by inducing or enhancing class I expression on tumor cells via secretion of IFN- γ (4).

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²These authors contributed equally to this work.

³To whom requests for reprints should be addressed, at Duke University Medical Center, MRSB 466, Box 2626, Durham, NC 27710. Phone: (919) 684-9949; Fax: (919) 681-7414; E-mail: j.vieweg@duke.edu.

⁴The abbreviations used are: DC, dendritic cell; hTERT, human telomerase reverse transcriptase; LAMP, lysosome-associated membrane protein; gp, glycoprotein; LCL, lymphoblastoid B-cell line; PBMC, peripheral blood mononuclear cell; TRAP, telomerase repeat amplification protocol; DOTAP, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate; ELISPOT, enzyme-linked immunospot; GFP, green fluorescence protein; IL, interleukin.

Cumulatively, these findings support the concept that in cancer immunotherapy not only specific HLA class I-specific, but also HLA class II-restricted, epitopes of tumor antigens should be targeted. Unfortunately, this is largely hampered by the diversity of MHC class I and II alleles of individual patients as well as by the lack of defined HLA class II-restricted peptide epitopes.

One clinically applicable option for the generation of CD4⁺ T-cell responses *in vivo* is to use DCs pulsed with immunogenic proteins such as keyhole limpet hemocyanin, tuberculin, or tetanus toxoid along with tumor antigens, thereby enhancing primary antigen-specific T-cell responses (5, 6). The problem with this approach is that the activated/effect or helper CD4⁺ T cells provide only unspecific T-cell help during CTL priming at the lymph node draining the site of immunization. Moreover, antigen-specific CD4⁺ T cells will be absent at the tumor site, where they are critically needed to: (a) expand the activated/effect or CTLs; and (b) maintain an antitumor immune response by providing T-cell memory. Alternatively, it has been shown that MHC class II presentation of antigens can be greatly enhanced by using a sorting signal of the lysosome-associated membrane protein LAMP-1 that directs the endogenously expressed antigen into the lysosomal compartment and, to a lesser extent, into late endosomes (7). Routing of antigens into the MHC class II pathway resulted in enhanced presentation of CD4⁺ T cells, thereby enhancing the magnitude of primary T-cell responses *in vitro* (8) as well as antitumor immunity in tumor-bearing mice (9).

In this study, we sought to determine whether human DCs transfected with mRNA encoding a chimeric hTERT/LAMP-1 fusion protein are capable of enhancing the stimulation of hTERT-specific CD4⁺ T-cell responses, thereby constituting a useful strategy for immunotherapy of cancer. The hTERT/LAMP-1 template used for *in vitro* transcription was created by: (a) fusing the sequences encoding the lysosomal targeting signal of the human LAMP-1 protein to the COOH terminus of hTERT; and by (b) adding a sequence encoding the signal peptide of heat shock protein 96 (gp96) to the NH₂ terminus of the hTERT protein.

We show that transfection of DCs with hTERT/LAMP-1 RNA leads to enhanced stimulation of hTERT-specific CD4⁺ T cells without affecting intracellular generation and subsequent presentation of MHC class I epitopes, hence, generating a CTL response. These findings support the concept of using DCs transfected with the chimeric hTERT/LAMP-1 RNA in vaccine trials to generate CD4⁺ T-cell responses that may be required to stimulate and maintain an optimal hTERT-specific CD8⁺ CTL response in cancer patients.

MATERIALS AND METHODS

Cell Lines and Human Tissues. Human tumor cell lines that included SW620 (colon carcinoma), LNCaP (prostate carcinoma), MCF-7 (breast cancer), HepG2 (hepatocellular carcinoma), and K562 cells (chronic myelogenous leukemia) were obtained from the American Type Culture Collection, (Manassas, VA). The B-95-8 cell line that was used for production of Epstein-Barr virus was kindly provided by Dr. H. Heslop (Baylor College of Medicine, Houston, TX). Lymphoblastoid cell lines (LCLs) were established according to published protocols (10). Cell lines were maintained in complete RPMI 1640

supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies, Inc., Gaithersburg, MD). For HLA-A0201 typing, blood donors and cell lines were prescreened with the HLA-A2-specific antibody BB7.2 (American Type Culture Collection) by flow cytometry. HLA-A2-positive cells were subtyped by PCR as described previously (11). All human tissues or PBMCs were obtained after informed consent from patients according to an Institutional Review Board-approved protocol.

DC Generation from Peripheral Blood. For DC culture, we used techniques described previously (12). Briefly, leukapheresis-derived PBMCs were separated by density gradient centrifugation over Histopaque (Sigma Diagnostics, St. Louis, MO). The cells were then washed and resuspended in X-VIVO-15 medium (BioWhittaker, Walkersville, MD) and finally incubated for 2 h at 37°C to allow plastic adherence. Adherent cells were cultured in 6-well plates in 2 ml of X-VIVO-15 per well (10^6 cells/ml), supplemented with 2 mM L-glutamine (Life Technologies, Inc.), penicillin-streptomycin (100 IU/ml and 100 µg/ml; Life Technologies, Inc.), 800 IU/ml granulocyte/macrophage-colony stimulating factor, and 500 IU/ml IL-4 (R&D Systems, Minneapolis, MN). After 7 days of culture, cells were harvested and phenotypically characterized by flow cytometry to assure that they met the characteristic phenotype of immature DCs: MHC class I^{low}, MHC class II^{high}, CD80^{low}, CD86^{low}, CD83^{neg}, CD3^{neg}, CD14^{neg}, CD19^{neg}, and CD16/CD56^{neg}. DC preparations fulfilling these phenotypic criteria were used for subsequent RNA transfection.

Plasmid Constructs. The generation of plasmids pGEM4Z/A64 and pGEM4Z/GFP/A64 has been described previously (13). To generate pGEM4Z/hTERT/A64, we have subcloned the cDNA encoding hTERT into pGEM4Z/A64. The hTERT cDNA fragment was excised from the pGRN145 plasmid (GenBank accession number AF015950) using restriction site EcoRI and inserted into pGEM4Z/A64 that had been restricted with EcoRI. This fragment contains the 3397 nucleotide open reading frame of hTERT plus 3 nucleotides of 5' untranslated region and 6 nucleotides of 3' untranslated region, respectively. The *in vitro* transcribed RNA contains 58 additional nucleotides at the 5' end that consist of 7 nucleotides following the bacteriophage T7 polymerase transcription initiation site plus 51 nucleotides that are contributed by the multiple cloning site. At the 3' end, the RNA contains 47 nucleotides of the multiple cloning site, 6 nucleotides of the *PacI* site, the A64 polyadenylation sequence, and 4 nucleotides contributed by the *SphI* site. hTERT RNA can be generated using bacteriophage T7 RNA polymerase after linearizing pGEM4Z/hTERT/A64 with restriction endonuclease *SphI*. The *in vitro* transcribed hTERT mRNA has a size of 3528 nucleotides.

The plasmid pGEM4Z/hTERT/LAMP/A64 is a derivative of pGEM4Z/hTERT/A64. pGEM4Z/hTERT/A64 has been modified by: (a) deletion of the 167 NH₂-terminal amino acids of TERT; (b) NH₂-terminal fusion of 27 amino acids derived from human gp96 that represent the endosomal leader sequence; and (c) deletion of the hTERT stop codon and subsequently fusing 35 amino acids, comprising the transmembrane region as well as the lysosomal targeting sequence, of hLAMP-1 with the COOH terminus of hTERT. The resulting *in vitro* transcribed RNA contains 19 additional nucleotides (7 nucleotides vector sequence following the T7 transcription initiation site, 6 nucleotides of the *HindIII* site, and 6 nucleotides 5' untranslated region) at its 5' end. At the 3' end of the coding region for hTERT/LAMP-1 (3084 nucleotides), there are 48 nucleotides contributed by the multiple cloning site, 6 nucleotides contributed by the *PacI* site, 64 As representing the polyA stretch, and 4 nucleotides contributed by the *SphI* site. Accordingly, the length of *in vitro* transcribed hTERT/LAMP-1 RNA is 3225 nucleotides.

RNA Isolation and *In Vitro* Transcription of RNA. RNA was isolated from tissue or cells using the RNeasy kit according to the protocol provided by the manufacturer (Qiagen, Valencia, CA). Plasmids were linearized using restriction endonuclease *SphI* (Roche Diagnostics, Indianapolis, IN) and *in vitro* transcribed using the mMESSAGEmACHINE high yield capped RNA transcription kit (Ambion, Austin, TX) according to the manufacturer's manual. After digestion of the DNA template with DNase I, RNA was purified with an RNeasy kit (Qiagen, Valencia, CA).

RNA Transfection and Maturation of Cultured Autologous DCs. Transfection of serum-free cultured, immature DCs was performed in Opti-MEM medium (Life Technologies, Inc.). Briefly, the DCs were washed twice and resuspended in Opti-MEM medium at $1.6-4 \times 10^6$ cells/ml in 15-ml polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ). The cationic lipid *N*-(1-(2,3-dioleyloxy)propyl)-*N,N,N*-trimethylammonium methyl-

sulfate (DOTAP) (Roche) was used to deliver RNA into the cells. Five µg of *in vitro* transcribed RNA or total tissue RNA and 10 µg liters of DOTAP were mixed in a total volume of 500 µl liters of Opti-MEM in a 15-ml polystyrene tube at room temperature for 20 min. The RNA-lipid complex was added to the DCs in a total volume of 1 ml and incubated at 37°C, with occasional agitation, for 3 h. The cells were then washed and incubated in 2 ml of X-VIVO 15 serum-free medium containing granulocyte/macrophage-colony stimulating factor (800 units/ml) and tumor necrosis factor (200 units/ml; R&D Systems) for 24 h.

Real-Time Quantitative TRAP for the Detection of Telomerase Activity. Telomerase activity was determined by a modified TRAP as published previously (14). In this study, 0.5 µg of cell lysate was used for TRAP assays, and reactions were carried out in 1× SYBR green buffer (PE Applied Biosystems, Branchburg, NJ). Real-time PCR was performed using the ABI-PRISM 7700 thermocycler. For detection of telomerase amplicons by gel electrophoresis, 1 µg of primer TS was 5'-end labeled using bacteriophage T4 polynucleotide kinase and 10 µCi of [γ -³²P]ATP and included in PCR reactions. The specific activity of the labeled primer was 5×10^6 µCi of DNA. PCR reactions were subjected to electrophoresis on nondenaturing 12% polyacrylamide gels (acrylamide:bisacrylamide, 38:2) at 10 V/cm in 90 mM Tris-borate, 2 mM EDTA. After electrophoresis, PCR products were detected by autoradiography overnight.

Western Blot Analysis. For detection of hTERT and hTERT/LAMP-1 in transfected DCs or tumor cell lines, 100 µg of the respective cell lysates were subjected to electrophoresis on 6% SDS polyacrylamide gels. The separated proteins were then electrotransferred to 0.45 µm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) and incubated for 2 h at room temperature with hTERT-specific rabbit polyclonal antibody H-231 (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200 in PBS supplemented with 5% nonfat dry milk (Nestle, Solon, OH) and 0.02% Tween 20 (EM Science, Gibbstown, NJ). Filters were washed three times with PBS, 0.02% Tween 20 and twice with 50 mM Tris, 150 mM sodium chloride, pH 7.5 (TN). As secondary antibody, horseradish peroxidase-coupled antirabbit IgG sc-2030 (Santa Cruz Biotechnology) was used at a dilution of 1:5000 in TN, 5% nonfat dry milk. After 1 h at room temperature, filters were washed three times in TN, and protein bands were detected using the Western Lightning Chemiluminescence kit (Perkin-Elmer Life Sciences, Boston, MA), according to the manual provided by the manufacturer, and subjected to autoradiography for 10 min.

Proliferation Assay of CD4⁺ T Cells. Negative selection of CD4⁺ T lymphocytes was performed by magnetic bead separation (AutoMacs, Miltenyi, Germany). Purified cells were seeded into 96-well round-bottomed microplates at 10^5 cells/well. Cultured, irradiated (30 Gy) DCs (that had either been transfected with hTERT, hTERT/LAMP-1, or with a control RNA) were added to the T cells at various ratios in triplicates. The final volume of each well was adjusted to 200 µl liters with complete medium. Triplicate wells of T cells alone were used as the background control. After 3 days of culture, 1 µCi of [*methyl*-³H]thymidine (NEN, Boston, MA) was added to each well, and incubation was continued for an additional 18 h. Cells were collected onto glass fiber filters (Wallac, Turku, Finland) with a cell harvester, and uptake of thymidine was determined using a liquid scintillation counter.

Generation of hTERT-specific, Short-Term Cytotoxic T-Cell Lines Using hTERT or hTERT/LAMP-1 mRNA-transfected DCs. hTERT RNA, hTERT/LAMP-1 RNA, or control RNA-transfected DCs were used to generate hTERT-specific CTL lines. Transfected DCs were cocultured with autologous PBMCs (nonadherent fraction after adherence step for DC generation) at a DC:PBMC ratio of 1:10. Cells were restimulated twice, 7 days apart, and IL-2 (10 units/ml) was added after 7 days. After 20 days of culture, effector cells were harvested for microcytolytic assays. As determined by flow cytometry, 80% of cells were CD3⁺, and ~30% exhibited a CD8⁺ phenotype.

Cytolytic Assays. Target cells (1×10^6) were labeled with 100 µCi of Na₂[⁵¹CrO₄] (NEN) in 200 µl of complete RPMI 1640 for 1 h at 37°C in 5% CO₂. After three washes, 5×10^3 ⁵¹Cr-labeled target cells were incubated in 200 µl of complete RPMI 1640 for 4 h at 37°C in 5% CO₂ at different E:T ratios. Then, 50 µl of supernatant were harvested, and the release of ⁵¹Cr was measured with a gamma counter. Antibody blocking experiments were performed at an E:T of 40:1 to determine whether cytosis was restricted by HLA class I or class II. Target cells were preincubated for 30 min with 20 g/ml of HLA-A,B,C-specific antibody W6/32 or HLA-DP-, HLA-DQ-, HLA-DR-

specific antibody CR3/43 (DAKO, Carpinteria, CA). Triplicate wells were averaged, and the percentage of specific lysis was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

IFN- γ Secretion Assays. For detection of IFN- γ secreted by activated CD4 $^{+}$ cells, the IFN- γ secretion assay detection kit (Miltenyi, Bergisch-Gladbach, Germany) was used according to the protocol provided by the manufacturer.

ELISPOT. After blocking wells with complete RPMI 1640, 1 \times 10 5 PBMCs or T cells and 1 \times 10 4 RNA-transfected DCs in 100 μ l of complete medium were added to each well of flat-bottomed, 96-well nitrocellulose plates (Multiscreen-IP; Millipore, Bedford, MA) precoated with 2 μ g/ml of IFN- γ capture antibody (Endogen, Woburn, MA). Plates were incubated for 20 h at 37°C, and after washing, biotinylated IFN- γ detection antibody (0.2 μ g/ml; Endogen) was added to each well. Wells were incubated for an additional 2 h at room temperature, then incubated with streptavidin-alkaline phosphatase (1 μ g/ml; Sigma), and developed with substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After washing, spots were counted using an automated Zeiss KS Elispot Compact reader (Carl Zeiss, Inc., Minneapolis, MN).

RESULTS

Generation of the Chimeric gp96/TERT/LAMP-1 Molecule. To target hTERT, a nuclear and cytosolic protein, to the endosomal and lysosomal cellular compartments, it was first necessary to place a signal peptide at the NH₂-terminus of the hTERT sequence to ensure efficient translocation into the endoplasmic reticulum. The transmembrane domain and cytoplasmic tail of human LAMP-1, including the tyrosine-based sorting motif YQTI, were added to the COOH terminus of hTERT, because these sequences have been shown to allow direct endosomal/lysosomal targeting via the *trans*-Golgi network by bypassing the plasma membrane (15). The DNA fragments that encode the NH₂-terminal signal peptide sequence (amino acids 1–27) of heat shock protein 96 (gp96) and the endosomal/lysosomal targeting signal (amino acids 382–408) of LAMP-1 were ligated into restriction sites present in the hTERT cDNA. Insertion of these constructs into the plasmid pGEM4Z-A64 (13) allowed *in vitro* transcription of a polyadenylated hTERT RNA and gp96/hTERT/LAMP-1 (hTERT/LAMP) RNA under control of the bacteriophage T7 promoter. Fig. 1 shows a diagram of the final constructs (*A*), as well as a denaturing agarose gel electrophoresis (*B*) of the *in vitro* transcribed RNA species used in our study.

hTERT Activity and hTERT Protein Expression in hTERT and hTERT/LAMP-1 RNA-transfected DCs. We next performed experiments to define the optimal conditions for transfection of monocyte-derived human DCs with *in vitro* transcribed hTERT mRNA. As a measure of transfection efficacy, we determined the expression levels of enzymatically active hTERT protein by these cells after maturation by using a modified real-time PCR-based TRAP. As positive or negative controls, we included the human prostate carcinoma cell line LNCaP (hTERT $^{+}$), patient-derived bone marrow and dermal tissues, as well as cells with reportedly low or absent hTERT expression including benign renal tissues, adrenal cells, or DCs transfected with GFP RNA. As shown in Fig. 2A, lipofection of DCs with hTERT mRNA consistently resulted in high endogenous levels of functional hTERT protein within transfected cells. hTERT activity in hTERT RNA-transfected DCs corresponded to 25 \pm 2.8% of the hTERT activity present in LNCaP cells, whereas bone marrow and dermal tissues exhibited relatively low levels of hTERT activity with 2.5 and 2.1%, respectively. Expectedly, only background levels (0.1–0.2%) of hTERT activity could be detected in benign renal tissue, adrenal cells, and GFP RNA-transfected DCs. Because SYBR green was used in our assays, which may nonspecifically bind to double-

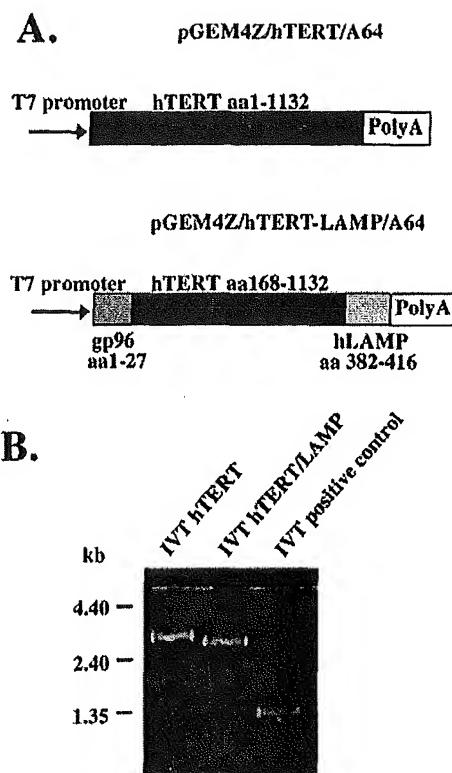


Fig. 1. Schematic diagram of the constructs pGEM4Z/hTERT/A64 and pGEM4Z/hTERT-LAMP/A64 and *in vitro* transcription of hTERT and hTERT/LAMP RNA. *A*, the complete coding region of hTERT, amino acids (aa) 1–1132, was cloned into the plasmid pGEM4Z/A64. The hTERT sequence was modified by adding amino acids 1–27 of human gp96 to the NH₂ terminus, by deleting amino acids 1–167 of hTERT and by fusing a DNA sequence encoding amino acids 383–416 of hLAMP-1 to the COOH terminus. PolyA indicates a synthetic poly adenosine stretch provided by pGEM4Z/A64. *B*, both constructs were *in vitro* transcribed (*IVT*), and the resulting RNA species were subjected to denaturing agarose gel electrophoresis and stained with ethidium bromide. As controls for the *in vitro* transcription reactions, an unrelated GFP-DNA (*IVT* positive control) or no DNA (*IVT* negative control) was used. The size of the resulting RNA species is indicated in kb.

stranded DNA, we confirmed that the fluorescence signals detected in our assays actually resulted from amplified hTERT products rather than from primer dimer artifacts, which could be detected only using more than 35 PCR cycles. Therefore, PCR reactions were performed in the presence of one 5'-³²P-labeled primer, and the amplified gene products were subjected to PAGE, as shown in Fig. 2B. The typical ladders of amplicons indicating elongation of the DNA template by hTERT activity were observed for cell extracts from LNCaP cells as well as DCs transfected with hTERT RNA but not for cell extracts obtained from heat-inactivated LNCaP or DCs treated with liposomes only, thus confirming the specificity of the modified real-time PCR-based TRAP assay. Unfortunately, this analysis cannot be applied to determine the level of hTERT/LAMP-1 protein in transfected DCs because even minor modifications of the NH₂ terminus of hTERT, such as point mutations, have been shown to completely abrogate hTERT activity (16). Therefore, we analyzed and compared protein expression by hTERT RNA- and hTERT/LAMP-1 RNA-transfected DCs in Western blots probed with anti-hTERT antibody (Fig. 2C). Taking into account the quantitative limitations of Western blotting, we determined that reproducibly similar amounts of hTERT and hTERT/LAMP-1 were expressed in DCs transfected with the corresponding RNA via lipofection.

In summary, we have shown that lipofection of immature, monocyte-derived DC with plasmid-derived hTERT mRNA resulted in the

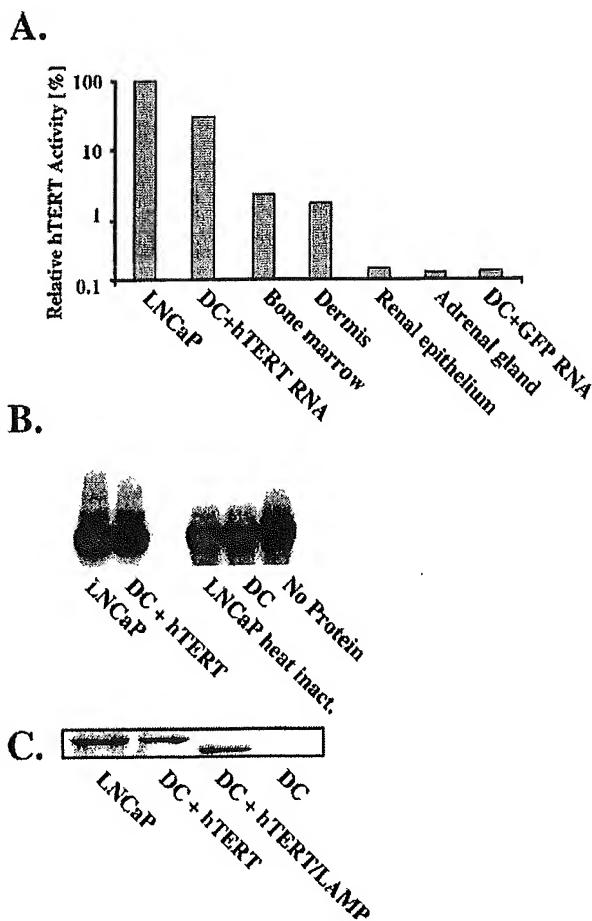


Fig. 2. hTERT protein activity in DCs transfected via lipofection and in tissue samples as determined by real-time PCR TRAP assays. *A*, expression of hTERT activity in hTERT RNA-transfected DCs, and various tissues was measured using a modified real-time TRAP assay. The measured hTERT activity levels were expressed as a percentage of activity found in the reference cell line LNCap (please note that the *ordinate* is in logarithmic scale). *B*, real-time PCR reactions were performed including a 5'-³²P-labeled primer and subjected to nondenaturing PAGE. PCR products were detected by autoradiography (*LNCap heat inact.*, cell extracts were heated for 5 min at 96°C). *C*, Western blot analysis of LNCap, DCs transfected with hTERT RNA or hTERT/LAMP1 RNA, and DCs treated with liposome only. Blots were probed with hTERT-specific polyclonal antibody, and specific bands were detected by enhanced chemiluminescence.

expression of high levels of intracellular and enzymatically active hTERT protein.

Evaluation of hTERT-specific T-Cell Responses. To assess and compare the efficacy between hTERT RNA- and hTERT/LAMP-1 RNA-transfected DCs to stimulate hTERT-specific T-cell responses *in vitro*, we first analyzed the frequencies of hTERT-specific T cells that could be expanded from the PBMCs of cancer patients using an IFN- γ ELISPOT assay. The goal of these studies was to measure the hTERT-specific T-cell reactivities directed against the entire hTERT molecule, which potentially included both hTERT-specific CD8 $^{+}$ CTLs as well as CD4 $^{+}$ T helper cellular subsets. Furthermore, we sought to determine whether RNA-transfected DCs could serve as stimulators in ELISPOT assays, hence allowing the determination of polyclonal T-cell reactivities against hTERT across a broad spectrum of HLA haplotypes.

In the experiment shown in Fig. 3, IFN- γ ELISPOT was performed using effector cells in the form of: (a) PBMCs (control); or of T cells stimulated with (b) hTERT RNA; or (c) hTERT/LAMP-1 RNA-transfected DCs. The following cells were used as stimulators: (a)

PSA RNA-transfected DCs (control RNA); (b) hTERT RNA-transfected DCs; and (c) hTERT/LAMP-1 RNA-transfected DCs. We found that effector cells stimulated by hTERT RNA- or hTERT/LAMP-1 RNA-transfected DCs produced significantly higher numbers of IFN- γ -positive spots than unstimulated PBMCs from the same patient. The exquisite antigen specificity of the hTERT-specific T cells was further shown by the fact that incubation of hTERT-specific effector cells with PSA-expressing targets resulted only in background spot levels.

In summary, these data demonstrate that hTERT RNA- or hTERT/LAMP-1 RNA-transfected DCs are similarly effective to stimulate hTERT-specific T cells from PBMCs with calculated frequencies of greater than 1:300 after two restimulation cycles using hTERT RNA- or hTERT/LAMP RNA-transfected DCs, respectively.

Killing of Tumor Targets by hTERT-specific CTLs. An important requirement prior to considering hTERT RNA-transfected DCs in immunotherapy clinical trials is to not only determine the presence but also the effector function of the DC-induced hTERT-specific CTLs. Therefore, we analyzed whether hTERT RNA-transfected DCs are capable of stimulating CTLs from PBMCs that specifically recognize and lyse hTERT-overexpressing targets as well as tumor targets in cytolytic assays. In these experiments, hTERT RNA-transfected DCs were generated from the PBMCs of an HLA-A0201 $^{+}$ patient and used to stimulate CTLs *in vitro*. After two restimulation cycles, effector cells were analyzed in standard cytotoxicity assays. The experiments shown in Fig. 4 demonstrate that hTERT RNA-transfected DCs are capable of stimulating T cells, which recognized and lysed the hTERT-overexpressing HLA-A0201 $^{+}$ tumor cell lines LNCaP, SW620, HepG2, and MCF-7 with similar or even higher efficacy than autologous hTERT RNA-transfected DC targets (Fig. 4, *A* and *B*). Moreover, autologous B-LCLs (hTERT $^{+}$), derived from a lymphoblastoid B-cell line, were efficiently lysed, whereas control targets in the form of: (a) autologous DCs transfected with GFP RNA; (b) natural killer cell-sensitive K562 cells; and (c) allogeneic DCs were not killed. On the basis of these experiments, it appears that tumor cell lysis was antigen-specific and mediated by CTLs but not by alloreactive cells or natural killer cells.

To gain preliminary evidence as to whether autoimmunity could represent a limiting factor for using hTERT RNA-transfected DCs in immunotherapy trials, we tested whether the hTERT-specific CTLs are not only capable of killing tumor targets but also of recognizing

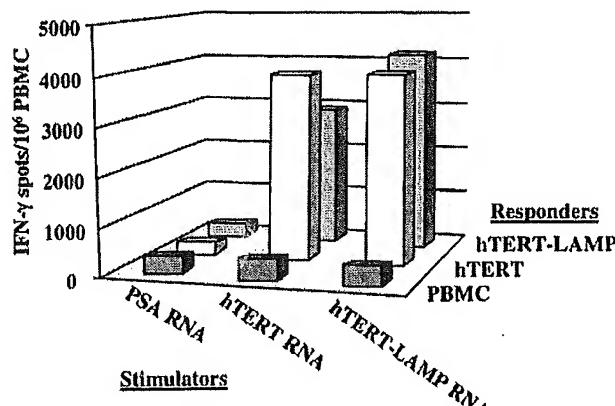


Fig. 3. IFN- γ ELISPOT assay to determine frequencies of hTERT-specific T cells. Effector cells were generated from PBMCs after two restimulation cycles with either hTERT or hTERT/LAMP RNA-transfected DCs. These cells were then incubated with specific (hTERT RNA- or hTERT/LAMP RNA-transfected DCs) or control stimulator cells [DCs transfected with prostate-specific antigen (PSA) RNA] overnight, and numbers of IFN- γ -secreting cells were calculated using an automated ELISPOT reader.

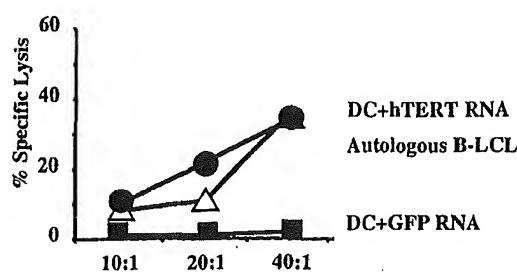
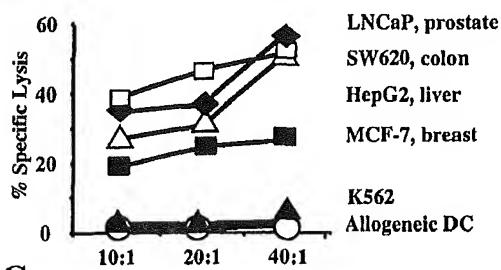
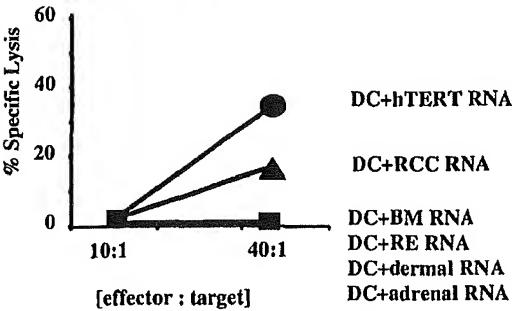
A. Stimulators: DC+hTERT RNA**B.****C.**

Fig. 4. Cytolytic assays for hTERT-specific CTLs. *A*, killing of an autologous, TERT⁺ lymphoblastoid B-cell line (*B-LCL*) and of autologous hTERT RNA-transfected DCs by hTERT-specific CTLs. As a control for antigen specificity, DCs transfected with GFP RNA were used as targets. *B*, lysis of hTERT-expressing HLA-A2⁺ tumor cell lines LNCaP, SW620, HepG2, and MCF-7 by hTERT-specific CTLs. As negative controls, HLA-class I-negative K562 cells, highly sensitive *in vitro* target for natural killer cells, and allogeneic DCs were included in our assays. *C*, DCs were transfected with the following RNA species: hTERT RNA, autologous renal cell carcinoma RNA (*RCC RNA*), bone marrow RNA (*BM RNA*), normal renal epithelium RNA (*RE RNA*), RNA isolated from dermal tissue (*dermal RNA*), and RNA isolated from adrenal gland (*adrenal RNA*), respectively. Transfected DCs were used as targets for hTERT-specific CTLs in cytolytic assays at E:T ratios of 10:1 and 40:1.

antigens expressed by normal tissues. In these experiments, we used a similar experimental setup as described previously (17). hTERT RNA-transfected DCs were used to stimulate CTLs, which were then analyzed for their ability to specifically kill hTERT-expressing tumor targets as well as DC targets transfected with normal tissue RNA extracted from dermal tissues, bone marrow, adrenal gland, or normal kidney (Fig. 4C). Similarly to the experiments shown in Fig. 4, *A* and *B*, the hTERT-specific CTLs were capable of lysing their cognate targets (hTERT RNA-transfected DCs) and also tumor targets in the form of renal tumor RNA-transfected DCs, albeit less efficiently, whereas no cytolysis of normal tissue targets was observed. In conclusion, these experiments demonstrate that hTERT RNA-transfected DCs are effective to stimulate antigen-specific T-cell responses *in vitro*. The hTERT-specific CTLs were capable of lysing tumor targets and did not cross-react with antigens expressed by benign tissues,

suggesting that vaccine-induced autoimmunity may not be a serious issue with this approach.

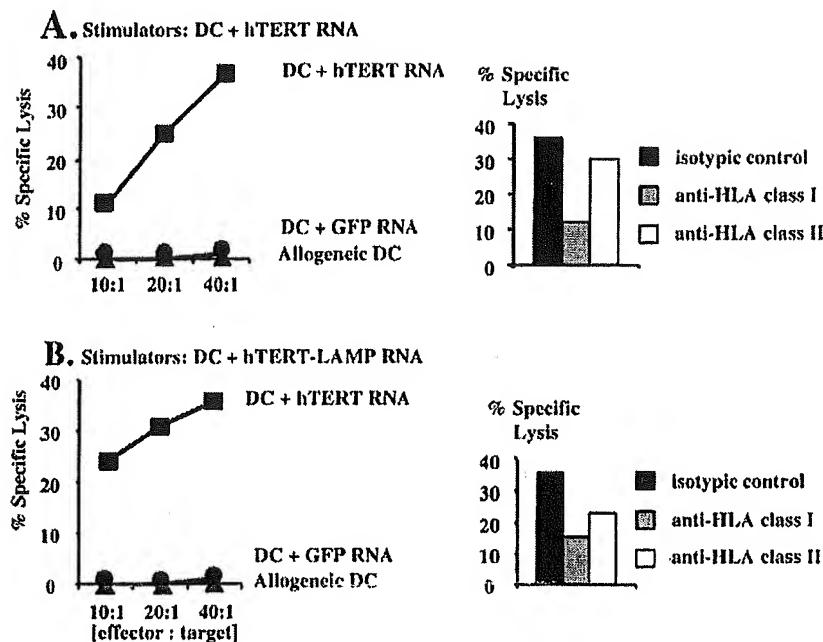
Specific Killing of hTERT-Expressing Targets by hTERT/LAMP-1-specific CTLs. We next analyzed whether hTERT/LAMP-1 RNA-transfected DCs are as effective as hTERT RNA-transfected DCs in stimulating antigen-specific and functionally active CTLs *in vitro*. Both hTERT RNA- and hTERT/LAMP-1 RNA-transfected DCs were generated from the PBMCs of an HLA-A0201⁺ patient and used to stimulate CTLs from PBMCs. After two restimulation cycles, effector cells were analyzed in cytolytic assays. As shown in Fig. 5, both the hTERT and the hTERT/LAMP-1-specific CTLs were equally effective in recognizing and lysing hTERT RNA-transfected DCs, whereas control targets in the form of GFP RNA-transfected autologous DCs or allogeneic DCs were not lysed. Interestingly, antibody blocking experiments reproducibly revealed that a considerable portion of the lytic activity of hTERT/LAMP-1-specific CTLs could be inhibited by anti-HLA class II (DP, DQ, and DR) antibody (Fig. 5B, right panel), which may indicate that not only class I-restricted CTLs but also CD4⁺ T cells with cytotoxic activity (8, 18) were generated using hTERT/LAMP-1 RNA-transfected DCs. In addition, no lysis of allogeneic DC targets was observed, suggesting that lysis of hTERT RNA-transfected DC targets was MHC class II restricted and mediated by the granzyme/perforin pathway rather than by unspecific Fas/Fas ligand- or tumor necrosis factor-related apoptosis-inducing ligand-mediated killing.

In summary, these experiments demonstrate that hTERT and hTERT/LAMP-1-transfected DCs are equally effective to stimulate CTLs *in vitro* that are capable of lysing hTERT-expressing cellular targets. Also, hTERT-specific CD4⁺ T cells may contribute to the antigen-specific recognition and killing of target cells *in vitro*.

DCs Transfected with hTERT/LAMP-1 RNA Improve CD4⁺ T-Cell Proliferation. The main objective of generating the chimeric hTERT/LAMP-1 construct was to route hTERT gene products into the class II presentation pathway, thereby eliciting an enhanced CD4⁺ T-helper response. Improved CD4⁺ T-cell induction was indirectly suggested by the pronounced blocking of the cytolytic activity of hTERT/LAMP-1-specific CTLs by anti-HLA class II antibodies, indicative for the presence of cytolytic CD4⁺ T cells (see Fig. 4B). As a more direct measure for MHC class II presentation, we next used standard proliferation assays to determine and compare the antigen-specific proliferation of CD4⁺ T cells generated by stimulation with hTERT RNA- or hTERT/LAMP-1 RNA-transfected DCs. PBMCs were stimulated once using DCs transfected with the modified (hTERT/LAMP) and unmodified (hTERT) RNA. CD4⁺ T cells were then isolated via magnetic bead separation and analyzed in standard proliferation assays. As shown in Fig. 6A, hTERT/LAMP-1 RNA-loaded DCs were reproducibly superior in stimulating CD4⁺ T-cell proliferation than unmodified hTERT mRNA-loaded DCs, whereas DCs transfected with GFP RNA demonstrated only background levels of CD4⁺ T-cell proliferation.

To provide a second line of evidence on LAMP-induced CD4⁺ T-cell stimulatory capacity, we measured the efficacy of hTERT and hTERT/LAMP-1 RNA-transfected DCs to expand memory T cells using flow cytometric detection of the Th1-type cytokine IFN- γ and the Th2-type cytokine IL-4 secreted by activated CD4⁺ T cells. Similar to the previous experiments, T cells were stimulated from PBMCs using hTERT RNA and hTERT-LAMP-1-RNA-transfected DCs. Stimulated CD4⁺ T cells were then isolated by magnetic bead separation and analyzed by flow cytometry for secretion of intracellular IFN- γ and IL-4. As shown in Fig. 6B, hTERT/LAMP-1 RNA-loaded DCs consistently stimulated higher frequencies of IFN- γ but not of IL-4 (0.5–1.2%; data not shown)-secreting CD4⁺ T cells than hTERT RNA-transfected DCs, regardless of whether passive pulsing

Fig. 5. Cytolytic assays for hTERT- and hTERT/LAMP-specific CTLs. Killing of hTERT RNA-transfected DCs by hTERT-specific (*A, left*) or hTERT/LAMP-specific (*B, left*) CTLs. In contrast, DCs transfected with GFP RNA or allogeneic DCs were not lysed, indicating antigen-specific cytotoxicity. In both representative experiments, specific lysis (E:T ratio, 40:1) was significantly inhibited by preincubation of DC targets with HLA class I-specific antibody (anti-HLA class I A, B, C) but to a much lesser extent by HLA class II-specific antibody (anti-HLA class II DP, DQ, DR), suggesting that lysis was predominantly mediated by hTERT-specific CTLs.



with "naked" RNA (*left panels*) or lipofection (*right panels*) was used for DC transfection.

The results outlined in Fig. 6 suggest that, as hypothesized, hTERT transcripts containing the endosomal/lysosomal targeting sequence of LAMP are, in fact, superior to unmodified hTERT RNA in stimulating hTERT-specific CD4⁺ T-cell responses *in vitro*.

DISCUSSION

The overall goal of this study was to develop a DC-based vaccination strategy designed for the concomitant induction of hTERT-specific CD8⁺ and CD4⁺ T-cell responses, both of which are considered critical components for potent and durable immune responses. We show that both hTERT RNA- and hTERT/LAMP-1 RNA-transfected DCs are remarkably effective in priming hTERT-specific CTLs *in vitro* that were capable of recognizing and lysing tumor targets. As hypothesized, DCs transfected with hTERT/LAMP-1 RNA stimulated: (*a*) significantly higher hTERT-specific CD4⁺ T-cell proliferation; and (*b*) stimulated 50% higher numbers of IFN- γ -secreting CD4⁺ T helper cells than DCs transfected with unmodified hTERT RNA, thereby potentially augmenting the cytotoxic T-cell response against hTERT-expressing targets.

hTERT, the core catalytic subunit of the human telomerase holoenzyme, is considered to be an attractive antigenic target for most cancers, because hTERT is overexpressed in ~90% of all solid tumors (19, 20). In addition, the critical importance of hTERT in allowing uninhibited growth of malignant tumors has been shown in recent studies that demonstrated that low-level expression of mutant hTERT template RNA results in decreased tumor cell proliferation and increased cell death, even in the absence of telomere shortening (21).

At first glance, the increases in LAMP-driven stimulation of CD4⁺ T cells observed in this study appear to be modest. This, however, was not entirely unexpected, because channeling of endogenous antigens into MHC class II presentation has been shown to be less efficient than loading of class II molecules with exogenous antigens (22). In addition, in previous studies reporting the successful LAMP-driven stimulation of CD8⁺ and CD4⁺ T cell responses, viral or neoantigens, but not tumor antigens for which peripheral tolerance has been estab-

lished, were used (23–27). On the other hand, the increases in hTERT-specific CD8⁺ and CD4⁺ T cells in our study are consistent with those shown in a prior report in which carcinoembryonic antigen-LAMP RNA-transfected DCs were used for the generation of primary T-cell responses (8). Unexpectedly, the proliferation and cytokine secretion assays shown in Fig. 6 suggested significant MHC class II presentation by DCs transfected with unmodified hTERT RNA, regardless of whether naked RNA or cationic lipids were used for RNA delivery. Furthermore, IL-2 was dispensable to prime hTERT-specific CTLs, both for hTERT-specific and hTERT/LAMP-1-specific T-cell lines, suggesting that MHC class II presentation and, therefore, CD4⁺ T-cell help was provided. MHC class II presentation by DCs that have been transfected with RNA-encoded antigens has been observed in two prior studies (8, 28) and has been attributed to the fact that antigens in the form of membrane or secretory proteins were used that gain access to the class II pathway. The mechanisms by which RNA-encoded hTERT, a cytosolic and nuclear protein, may find access into the MHC class II presentation pathway are unknown thus far and currently under investigation in our laboratory.

hTERT is not only expressed in cancer cells but also, at significantly lower levels, in embryonic cells and in adult male germ-line cells. It is, however, undetectable in normal somatic cells, except for proliferative cells of renewal tissues, *i.e.*, hematopoietic stem cells, activated lymphocytes, basal cells of the epidermis, proliferative endometrium, and intestinal crypt cells (29). This fact raises the question of whether vaccination of cancer patients with hTERT/LAMP-1 RNA carries the risk of inducing autoimmune responses with pathological consequences. (30, 31). On the basis of the results provided in Fig. 4C, we expect this risk to be minimal, because hTERT-specific CTLs recognized and lysed only hTERT-overexpressing tumor targets but not target cells expressing normal tissue antigens. Although some concerns may remain for male germ-line cells that have high hTERT activity, their sensitivity to T-cell attack is expected to be low because these tissues generally fail to express HLA class I molecules, an essential prerequisite for T-cell recognition. Interestingly, when we measured the levels of functional intracellular hTERT protein in various target cells, we found a strong correlation between hTERT

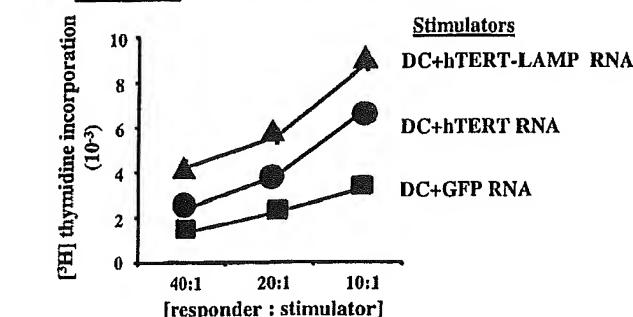
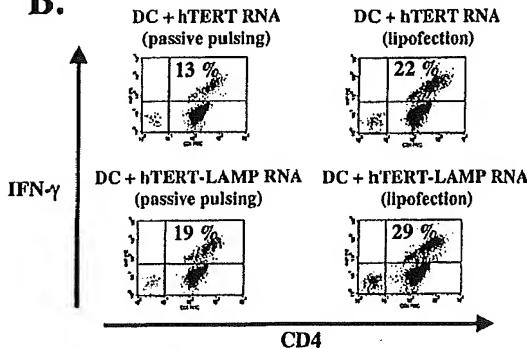
A. Responders: hTERT/LAMP CD4⁺ T cells**B.**

Fig. 6. Stimulation of hTERT-specific CD4⁺ T cells. PBMCs were stimulated once with hTERT RNA-transfected DCs. CD4⁺ cells were isolated by magnetic bead separation, and antigen-specific proliferation was determined upon restimulation using DCs transfected with hTERT RNA- or hTERT/LAMP RNA-transfected DCs as stimulator cells (*A*). Stimulators in the form of DCs transfected with GFP RNA were used as a control for unspecific T-cell activation. *B*, cytokine expression profile of hTERT/LAMP-specific CD4⁺ cells. PBMCs were restimulated twice with hTERT/LAMP RNA-transfected DCs and analyzed in IFN- γ secretion assays. DCs, passively pulsed with "naked" hTERT or hTERT/LAMP RNA or transfected with hTERT or hTERT/LAMP RNA via lipofection were used as stimulators in these assays.

protein expression and target cell recognition, suggesting that a threshold level of intracellular hTERT protein must exist to effect cellular lysis by hTERT-specific CTLs. Although our studies suggest that the expression levels of hTERT protein found in nonmalignant tissues are clearly below this necessary threshold level, further investigation is required to more accurately define the exact level of hTERT protein expression necessary for target cell recognition and lysis. To address this issue, studies are currently ongoing in our laboratory using cell lines that have been stably transfected with hTERT and express hTERT protein under the control of a regulatory promoter/enhancer element.

In conclusion, we show that DCs transfected with hTERT/LAMP-1 RNA represent a suitable vaccine strategy geared for the concomitant induction of hTERT-specific CD8⁺ and CD4⁺ T-cell responses. The data presented represent a scientific rationale to consider this strategy for further clinical investigation in subjects with hTERT-overexpressing malignancies.

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